

AMENDMENTS TO THE SPECIFICATION

Please cancel the amendments to the specification set forth in the September 21, 2005 Response and replace with the following amendments to the specification:

Please amend the paragraph on page 5, at lines 3 and 4 as follows:

Nucleotide and amino acid sequences are referred to by a sequence identifier, i.e. ~~<400>1~~, ~~<400>2~~ SEQ ID NO:1, SEQ ID NO:2, etc. A sequence listing is provided after the claims.

Please amend the paragraph on page 6, at lines 12-31 as follows:

Even still another aspect of the present invention contemplates a method for the prophylaxis or treatment of infection by a microorganism in a mammal, said microorganism substantially requiring exogenous iron, heme or porphyrin for growth or maintenance wherein said method comprises administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a molecule derived from said microorganism and having an HA2 domain and an HA2-binding moiety on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme and wherein said HA2 domain comprises:

- (i) an amino acid sequence substantially encoded by the nucleotide sequence set forth in ~~<400>5~~ SEQ ID NO:5 or a nucleotide sequence having at least about 40% similarity thereto or capable of hybridizing thereto under low stringency conditions; and/or
 - (ii) an amino acid sequence substantially as set forth in ~~<400>6~~ SEQ ID NO:6 or an amino acid sequence having at least about 40% similarity thereto or at least about 20% identity after optimum alignment with the same sequence;
- wherein said amino acid sequence is capable of interacting with an HA2-binding motif on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme.

Please amend the paragraph on page 7, at lines 1-19 as follows:

Even yet another aspect of the present invention provides a method for the prophylaxis or treatment of infection by a microorganism in a mammal, said microorganism substantially requiring exogenous iron, heme or porphyrin for growth or maintenance wherein said method comprises administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a molecule derived from said microorganism and having an HA2 domain and an HA2-binding moiety on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme and wherein said HA2 domain comprises:

- (i) an amino acid sequence substantially encoded by the nucleotide sequence set forth in ~~<400>5~~ SEQ ID NO:5 or a nucleotide sequence having at least about 40% similarity thereto or capable of hybridizing thereto under low stringency conditions; and/or
- (ii) an amino acid sequence substantially as set forth in ~~<400>6~~ SEQ ID NO:6 or an amino acid sequence having at least about 40% similarity thereto or at least about 20% identity after optimum alignment with the same sequence;

and wherein the HA2-binding motif comprises a moiety structurally or functionally homologous to substructure (Ia) of structure (I) below:

Please amend the paragraph on page 8 starting at line 10 and continuing on page 9 through line 3 as follows:

Still another aspect of the present invention contemplates a method for the prophylaxis or treatment of *P. gingivalis* infection or infection by a related microorganism in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a *P. gingivalis*-derived HA2- containing molecule comprising the amino acid sequence ALNPDNYLISKDVTG ~~<400>1~~ SEQ ID NO:1 or ALNPDNYLISKDVTGATKVKY ~~<400>8~~ SEQ ID NO:8 or an amino acid sequence having at least 40% similarity to ~~<400>1~~ or ~~<400>8~~ SEQ ID NO:1 or

SEQ ID NO:8 at least about 20% identity after optimum alignment with same sequence or an amino acid sequence encoded by the nucleotide sequence ~~<400>7~~ SEQ ID NO:7 or a nucleotide sequence having at least 40% similarity thereto or a nucleotide sequence capable of hybridizing thereto under low stringency conditions and an HA2-binding motif comprising and including propionic acid groups or anionic or salt forms thereof such as but not limited to the region defined by substructure (Ic) in Formula (I) on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme.

Please amend the Summary of Sequence Identifiers section on page 10 as follows:

SUMMARY OF SEQUENCE IDENTIFIERS

Sequence	Sequence Identifier
Amino acid marked for HA2 binding (peptide #1)	<400>1 <u>SEQ ID NO:1</u>
Forward primer to amplify HA2	<400>2 <u>SEQ ID NO:2</u>
Reverse primer to amplify HA2	<400>3 <u>SEQ ID NO:3</u>
Amino acid sequence of peptide #2	<400>4 <u>SEQ ID NO:4</u>
Nucleotide sequence encoding HA2 domain	<400>5 <u>SEQ ID NO:5</u>
Amino acid sequence of HA2 domain	<400>6 <u>SEQ ID NO:6</u>
Nucleotide sequence encoding <400>8 <u>SEQ ID NO:8</u>	<400>7 <u>SEQ ID NO:7</u>
Amino acid marked for HA2 binding (peptide #3)	<400>8 <u>SEQ ID NO:8</u>

Please amend the Brief Description of the Figures section starting on page 11 as follows:

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a diagrammatic representation of the domain structure and homologies between the gingipains, RgpA and Kgp. CAT represents putative catalytic domain and HA represents

putative hemagglutinin domains. Shaded areas represent regions of > 98% amino acid identity between the two gingipains. Fractions represent the degree of the identity for each RgpA domain.

Figure 2 Figures 2a and 2b ~~[[is a]] are graphical representation representations~~ showing hemoglobin binding by rHA2, RgpA, and Kgp. (2a): Microtiter wells were coated with hemoglobin then incubated with 3 fold dilutions of purified rHA2, 2.5 $\mu\text{g/ml}$ (diamonds), RgpA, 5 $\mu\text{g/ml}$ (circles), or Kgp, 5 $\mu\text{g/ml}$ (triangles). Association of rHA2 with hemoglobin was measured with mAb 5A1 followed by substrate development at 414 nm after binding of secondary anti-mouse alkaline phosphatase-conjugated antibody. (2b): Hemoglobin binding by native but not denatured gingipains. Wells were coated with hemoglobin then incubated overnight with dilutions of either Rgp-A (closed circles), Kgp (closed triangles), or RgpA denatured by boiling (open circles) or Kgp denatured by boiling (open triangles). For this experiment, native or denatured gingipains that bound to hemoglobin were recognized with mAb IIB2, which specifically detects both native and denatured gingipains. Primary antibody IIB2 was followed by substrate development at 414 nm after binding of secondary anti-mouse AP-conjugated antibody. Data are representative of three separate experiments.

Figure 3 Figures 3a and 3b ~~[[is a]] are graphical representation representations~~ showing binding of the HA2 domain to the heme moiety. (3a): Binding of rHA2 to dilutions of hemin (diamonds), hemoglobin (circles), or hemoglobin degraded by proteinase-K (triangles). Microtiter wells were coated with dilutions of samples then overnight binding of rHA2 to coated wells was detected with mAb 5A1 followed by substrate development at 414 nm after binding of secondary anti-mouse AP-conjugated antibody. The absence of contaminating protein within 90 μg of the hemin preparation and the absence of non-degraded subunits of hemoglobin remaining after proteinase-K treatment was verified by SDS-PAGE (data not shown). (3b): Binding of rHA2 to hemin. Microtiter wells were coated with hemin and overnight binding of rHA2 dilutions was detected with mAb 5A1 as above. Data are representative of two separate experiments.

Figure 4 Figures 4a and 4b ~~[[is a]] are graphical representation representations~~ showing inhibition of hemin- or hemoglobin-binding. Microtiter wells were coated overnight with hemin (panel a) or hemoglobin (panel b). rHA2 in *E. coli* lysate (100 fold dilution) (X), 65 ng/ml RgpA (circles) or 65ng/ml Kgp (triangles) were preincubated with dilutions of 300 μ M protoporphyrin IX for 1 hr then transferred to the ligand-coated plates for overnight incubation. Binding of rHA2 or the gingipains to coated wells was detected with mAb 5A1 or mAb IIB2, respectively, followed by substrate development at 414 nm after binding of secondary anti-mouse AP-conjugated antibody. Data are representative of two separate experiments. The absence of contaminating protein in a 90 μ g protoporphyrin IX preparation was verified by SDS-PAGE and by Coomassie dye binding.

Figure 5 Figures 5a, 5b and 5c ~~[[is a]] are diagrammatic representation representations~~ showing directed porphyrin-binding by rHA2. Microtiter wells were coated with 100 mM ethylene diamine (pH 4.7) then incubated with 90 μ g/ml hemin, protoporphyrin IX, or hematoporphyrin overnight in 50% dimethyl formamide in the presence (+) or absence (-) of 10 mM carbodiimide. Wells were washed 4 times with water then the amount of porphyrin bound to the wells was determined by absorbance at 414 nm (striped bars). Wells were blocked with PBS/Tween then incubated with 125 ng/ml rHA2 overnight. Binding of rHA2 to coated wells was detected with mAb 5A1 followed by substrate development at 414 nm after binding of secondary anti-mouse AP-conjugated antibody (solid bars). Error bars represent standard deviation of absorbance measurements. Diagrams of chemical structures for hemin, protoporphyrin IX, and hematoporphyrin are presented adjacent to corresponding data.

Figure 6 Figures 6a, 6b and 6c ~~[[is a]] are graphical representation representations~~ showing measurement of high-affinity binding of mAb 5A1 with rHA2 gingipains and gingipains from the culture supernatant. (6a): RgpA (circles), Kgp (triangles) or rHA2 in crude *E. coli* lysate (squares) were coated onto microtiter wells and incubated with serial dilutions of mAb 5A1. (6b): Dilutions of Rgp-A (open circles), Kgp (open triangles), or heat denatured Rgp-A (closed circles) or Kgp (closed triangles) were coated onto microtiter wells with 3 fold dilutions from 10 μ g/ml then incubated with mAb 5A1. (6c): Purified rHA2 (squares) or

purified high molecular-weight aggregates of gingipain domains isolated from culture supernatant (circles) were coated onto microtiter wells and incubated with serial dilutions of mAb 5A1. Data are representative of three separate experiments.

Figure 7 is a graphical representation showing immunoreactivity of synthetic peptides with mAb 5A1. ELISA demonstrating selective immunoreactivity of mAb 5A1 with peptide #1. Peptide #1 (squares) or peptide #2 (triangles) were coated onto microtiter plates at a concentration of 5 $\mu\text{g/ml}$ overnight then incubated with dilutions of mAb 5A1. Data are representative of two separate experiments.

Figure 8 Figures 8a, 8b and 8c ~~[[is a]] are graphical representation representations~~ showing expression of HA2-related immunoreactive hemoglobin-binding protein from *P. gingivalis*. Aliquots of *P. gingivalis* culture medium were removed daily during a period of 8 d and immediately separated into a cell pellet and culture supernatant then frozen until use. OD^{660} and purity of the culture were measured daily. The cell pellets were dispersed evenly into 1 ml of PBS/ N_3 . (8a) and (8b): Arg- and Lys-specific proteinase activities, respectively, of the cell-free culture supernatant (squares) and cellular fraction (triangles) were measured as described. Measurements of the cellular fractions were normalized to culture densities (OD^{660}) recorded daily. (8c): The HA2 domain (1/243 dilution, open squares) and HA2 domain associated with hemoglobin-binding (1/81 dilution, solid squares) in culture supernatants were measured by ELISA and ligand binding assay, respectively, as described. In *P. gingivalis* whole cell fractions, the HA2 domain (1/243 dilution, open triangles) and HA2 domain associated with hemoglobin-binding (1/9 dilution, solid triangles) were measured by ELISA and ligand binding assay, respectively, as described. Measurements of the cell-associated fractions were normalized to culture densities (OD^{660}) recorded daily. Corresponding background immunoreactivity with a murine anti-human CD-19 IgG was subtracted from each measurement. Data are representative of two separate experiments in which patterns of expression were similar.

Figure 9 Figures 9a, 9b, 9c, 9d and 9e ~~[[is a]]~~ are graphical representation representations of ligand binding to decreasing concentrations of rHA2. The ligands employed were: (a) deuteroporphyrin 1X 2,4 disulfonic acid (DDS); (b) deuteroporphyrin 1X 2,4 bisethylene glycol (DBEG); (c) dipyrrole 1 (C1; see Figure 10); (d) dipyrrole 2 (C2; see Figure 10); (e) dipyrrole 3 (C3; see Figure 10). Binding between the ligand and rHA2 was detected with monoclonal antibody (MAb) VAI followed by goat anti-mouse AP conjugate.

Figure 10 Figures 10a, 10b, 10c, 10d, 10e, 10f and 10g ~~[[is a]]~~ are diagrammatic representation representations of dipyrroles 1 (C1), 2 (C2) and 3 (C3) and the porphyrins protoporphyrin 1X (PPIX), deuteroporphyrin 1X 2,4 dihydrochloride (DDH), deuteroporphyrin 1X 2,4 disulfonic acid (DDS) and deuteroporphyrin 1X 2,4 bisethylene glycol (DBEG).

Figure 11 Figures 11a, 11b, 11c, 11d, 11e, 11f and 11g ~~[[is a]]~~ are graphical representation representations of showing competition between a ligand and hemoglobin (HG) for rHA2 binding. The ligands employed were: (a) protoporphyrin 1X (PPIX); (b) deuteroporphyrin 1X 2,4 dihydrochloride (DDH); (c) deuteroporphyrin 1X 2,4 disulfonic acid (DDS); (d) deuteroporphyrin 1X 2,4 bisethylene glycol (DBEG); (e) dipyrrole 1 (C1; see Figure 10); (f) dipyrrole 2 (C2; see Figure 10) and (g) dipyrrole 3 (C3, see Figure 10).

Please amend the paragraphs starting on page 17, at line 5 and continuing on page 18 through line 22 as follows:

A particularly preferred HA2 domain comprises the following amino acid sequence:

```
Ala Asp Phe Thr Glu Thr Phe Glu Ser Ser Thr His Gly Glu Ala Pro
Ala Glu Trp Thr Thr Ile Asp Ala Asp Gly Asp Gly Glu Gly Trp Leu
Cys Leu Ser Ser Gly Gln Leu Asp Trp Leu Thr Ala His Gly Gly Thr
Asn Val Val Ser Ser Phe Ser Trp Asn Gly Met Ala Leu Asn Pro Asp
Asn Tyr Leu Ile Ser Lys Asp Val Thr Gly Ala Thr Lys Val Lys Tyr
Tyr Tyr Pro Val Asn Asp Gly Phe Pro Gly Asp His Tyr Ala Val Met
Ile Ser Lys Thr Gly Thr Asn Ala Gly Asp Phe Thr Val Val Phe Glu
```

Glu Thr Pro Asn Gly Ile Asn Lys Gly Gly Ala Arg Phe Gly Leu Ser
Thr Glu Ala Asn Gly Ala <400>6 (SEQ ID NO:6)

or a sequence having at least about 40% similarity to at least about 10 contiguous amino acids thereof or at least about 20% identity after optimum alignment with the same sequence.

Alternative percentage similarities include at least about 50% or 60% or 70% or 80% or 90% or above. Alternative percentage identities include at least about 25% or 30%, 40%, 50%, 60%, 70%, 80% or 90% or above. An HA2 domain is also conveniently defined by being encoded by a sequence of nucleotides comprising the following sequence:

gca gac ttc acg gaa acg ttc gag tct tct act cat gga gag gca cca
gcg gaa tgg act act atc gat gcc gat ggc gat ggt gag ggt tgg ctg
tgt ctg tct tcc gga caa ttg gac tgg ctg aca gct cat ggc ggc acc
aac gta gta agc tct ttc tca tgg aat gga atg gct ttg aat cct gat
aac tat ctg atc tca aag gat gtt aca ggc gca acg aag gta aag tac
tac tat cca gtc aac gac ggt ttt ccc ggg gat cac tat gcg gtg atg
atc tcc aag acg ggc acg aac gcc gga gac ttc acg gtt gtt ttc gaa
gaa acg cct aac gga ata aat aag ggc gga gca aga ttc ggt ctt tcc
acg gaa gcc aat ggc gcc <400>5 (SEQ ID NO:5)

or a nucleotide sequence having at least 40% similarity to at least about 30 contiguous nucleotides thereof or a nucleotide sequence capable of hybridizing thereto under low stringency conditions. Alternative percentage similarities include at least about 50 or 60%, 70%, 80% or 90% or above.

Accordingly, another aspect of the present invention contemplates a method for the prophylaxis or treatment of infection by a microorganism in a mammal, said microorganism substantially requiring exogenous iron, heme or porphyrin for growth or maintenance wherein said method comprises administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a molecule derived from said microorganism and having an HA2 domain and an HA2-binding moiety on a

porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme and wherein said HA2 domain comprises:

- (i) an amino acid sequence substantially encoded by the nucleotide sequence set forth in ~~<400>~~5 SEQ ID NO:5 or a nucleotide sequence having at least about 40% similarity thereto or capable of hybridizing thereto under low stringency conditions; and/or
- (ii) an amino acid sequence substantially as set forth in ~~<400>~~6 SEQ ID NO:6 or an amino acid sequence having at least about 40% similarity thereto or at least about 20% identity after optimum alignment with the same sequence;

wherein said amino acid sequence is capable of interacting with an HA2-binding motif on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme.

Please amend the paragraph on page 21 starting at line 1 and continuing on page 22 through line 5 as follows:

Accordingly, another aspect of the present invention provides a method for the prophylaxis or treatment of infection by a microorganism in a mammal, said microorganism substantially requiring exogenous iron, heme or porphyrin for growth or maintenance wherein said method comprises administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a molecule derived from said microorganism and having an HA2 domain and an HA2-binding moiety on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme and wherein said HA2 domain comprises:

- (i) an amino acid sequence substantially encoded by the nucleotide sequence set forth in ~~<400>~~5 SEQ ID NO:5 or a nucleotide sequence having at least about 40% similarity thereto or capable of hybridizing thereto under low stringency conditions; and/or

In one embodiment, the antagonism results from inhibiting interaction between a region of surface exposed porphyrin and in particular heme comprising propionic acid groups or their anionic or salt forms such as but not limited to the region defined by sub-structure (Ic) and an HA2 containing molecule comprising an epitope capable of interaction with monoclonal antibody mAb 5A1 (see ref 34). In accordance with the present invention, mAb 5A1 interacts with an epitope defined by amino acid sequence ALNPDNYLISKDVTG <400>1 SEQ ID NO:1 or ALNPDNYLISKDVTGATKVKY <400>8 SEQ ID NO:8 an amino acid sequence having at least 40% similarity thereto or at least about 20% identity after optimum alignment with same sequence including an amino acid sequence defined by <400>1 or <400>8 SEQ ID NO:1 or SEQ ID NO:8 but which has single or multiple amino acid substitutions, deletions and/or additions.

Please amend the paragraphs on page 24, at lines 10-25 as follows:

The amino acid sequence defined by <400>1 or <400>8 SEQ ID NO:1 or SEQ ID NO:8 is not the porphyrin binding site but a useful marker for HA2.

Accordingly, another aspect of the present invention contemplates a method for the prophylaxis or treatment of *P. gingivalis* infection or infection by a related microorganism in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to antagonize the interaction between a *P. gingivalis*-derived HA2- containing molecule comprising the amino acid sequence ALNPDNYLISKDVTG <400>1 SEQ ID NO:1 or ALNPDNYLISKDVTGATKVKY <400>8 SEQ ID NO:8 an amino acid sequence having at least 40% similarity to <400>1 or <400>8 SEQ ID NO:1 or SEQ ID NO:8 or at least about 20% identity after optimum alignment with same sequence or an amino acid sequence encoded by the nucleotide sequence <400>7 SEQ ID NO:7 or a nucleotide sequence having at least 40% similarity thereto or a nucleotide sequence capable of hybridizing thereto under low stringency conditions and an HA2-binding motif comprising and including propionic acid groups or anionic or salt forms thereof such as

but not limited to the region defined by substructure (Ia) on a porphyrin-containing molecule such as but not limited to hemoglobin or a precursor form thereof or part thereof such as heme.

Please amend the paragraph starting on page 37 at line 25 and continuing on page 38 through line 19 as follows:

EXAMPLE 4

Expression and purification of rHA2

Forward and reverse primers (AACCTGCAGCGCGCAGACTTCACGG ~~<400>~~² SEQ ID NO:2 and GGAAGCCAATGGCGCCAAAAGATCTAGT ~~<400>~~³ SEQ ID NO:3) were designed to amplify the HA2 domain from the *P. gingivalis* Arg-gingipain-1 proteinase gene (Accession Number U15282). Restriction sites for *Pst*I and *Bgl*II were designed into the 5' ends of the primers to facilitate cloning. Digested PCR product was ligated into the QIAexpressionist type III construct providing a 6x-His tag on the COOH-terminus (Qiagen Corp., USA). Transformation of the ligated construct was performed by electroporation into *E. coli* NM522 cells. *E. coli* cultures were grown at 37°C to an OD⁶⁰⁰ = 0.6 then induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 6 hours. Cells were harvested and resuspended to 5 ml per gram wet weight in buffer A (8 M Urea, 0.1 mM NaH₂PO₄, 0.01 mM Tris-HCl, pH 7.9). The cells were stirred for 2 hrs at room temperature taking care to avoid foaming. This cell lysate was subjected to centrifugation at 31,000 g for 30 min at room temperature to pellet the cellular debris then the supernatant was subjected to ultracentrifugation at 130,000 g for 2 hrs. The clarified lysate was loaded onto a nickel-nitrilotriacetic acid column (Ni-NTA, Qiagen Corp., USA) pre-equilibrated with buffer A. The Ni-NTA column was washed with buffer A until baseline was reached. The protein was refolded on this column by running a linear gradient of urea from 8 M to 0 M in 20 mM Tris-HCl, 500 mM NaCl, 10% v/v glycerol, pH 7.9. The protein was then eluted with 50 mM Tris-HCl, 500 mM NaCl, 10% v/v glycerol, 250 mM imidazole, pH 7.9. The eluant was diluted 100 fold in 50 mM sodium acetate buffer, pH 5.5 and applied to a hemoglobin-agarose column pre-equilibrated with the dilution buffer. After loading, the column was washed with

the same buffer until baseline was reached then the hemoglobin-binding protein was eluted with 50 mM tris-HCl, pH 9. Protein concentrations were determined by Coomassie dye binding using bovine serum albumin as a standard.

Please amend the paragraph on page 40, lines 26-30 as follows:

Peptides were synthesized by Chiron Mimotopes (Victoria, Australia) with terminal amines and carboxylic acids. Peptide #1 sequence was ALNPDNYLISKDVTG <400>1 SEQ ID NO:1. Peptide #2 sequence was GEAPAEWTTIDADGDGQGWL <400>4 SEQ ID NO:4. Peptide #3 is ALNPDNYLISKDVTGATKVKY <400>8 SEQ ID NO:8. The latter amino acid sequence is encoded by the nucleotide sequence set forth in <400>7 SEQ ID NO:7.

Please amend the paragraph on page 44, lines 7-15 as follows:

Using linear synthetic peptides, the epitope of mAb 5A1 was determined to be associated with the peptide ALNPDNYLISKDVTG <400>1 SEQ ID NO:1 ($K_d = 3.8$ nM) which represents amino acids #1215-1229 of the translated Kgp within the HA2 domain (Figure 7, peptide #1). Dot blot analysis on PVDF membrane confirmed the unique immunoreactivity of this peptide with mAb 5A1. Similar results were obtained with peptide #3 <400>8 SEQ ID NO:8. A search of SwissProt database for the linear sequence of peptide #1 or GENBANK® database using the deduced nucleic acid sequence of this epitope resulted in no molecules with perfect homology to the peptide other than the gingipains and HagA, a large hemagglutinin with regions of identity to the entire HA2 domain.